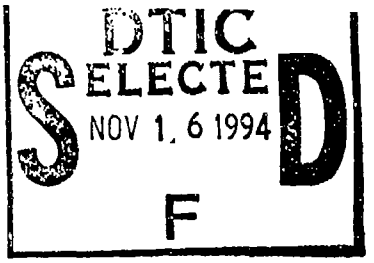

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Progress Report
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I. Work Summary

We continue to push ahead. In the course of our experiments over the last 4 months, two issues have emerged that we feel are important to resolve and to which we have devoted considerable effort. The first issue relates to the exact amino acid sequence of the LBP peptide that is optimal for coupling. This knowledge is crucial in order to scale up and produce conjugates for testing in vivo. The second issue, which is related, concerns the number of peptides/IgG that will be necessary for retaining activity in the bloodstream. These issues, and our approach to solve them, are discussed below.

So as not to lose time while awaiting a large batch of LBP peptide-IgG with the optimum sequence, we have started to study the stability of peptide-IgG conjugates in whole blood using the LBP peptides that we have in hand and a similar LPS binding peptide, CAP18. We hope that these experiments will save us time once we have the optimum LBP sequence and we are ready to scale up.

A. Progress on Specific Aim #1

At the start of the summer we were on the verge of submitting our manuscript on the exact peptide sequence of LBP that was necessary for binding and neutralization. However, we were bothered by something that we noted when we analyzed the data concerning the ability of the peptides to block LPS binding to the LPS receptor on cells (CD14). The peptides that we had constructed with a terminal cysteine residue on the carboxy terminal were more active than a series of control peptides that were missing the cysteine residue. In addition, some peptides with an additional 4 amino acids on the carboxy terminal end also had higher activity. These findings caused us to review all of our prior LPS binding and neutralization data.

Ordinarily cysteine is involved in forming the structure of disulfide bridges. Cysteine is usually not an amino acid that accounts for activity per se. We constructed our most active peptides with a terminal cysteine in order to couple the peptides to IgG via a disulfide bond. Thus, there was a general trend that the peptides that we had worked with the most had a terminal cysteine for coupling (in particular different lots of LBP76-102C and LBP86-102C). Since the LBP-IgG conjugates that we constructed have activity, we had not designed specific experiments to test for the activity of the cysteine. In addition, some lots of a slightly longer peptide (LBP86-106) had somewhat higher activity

in some of the assays on a molar basis than LBP86-102C. Furthermore, the single construct that we had of LBP86-102 (without the terminal cysteine) bound LPS well, but blocked binding to CD14 only modestly. Since cysteine can spontaneously form disulfide bridges in the oxidized state, these findings raised the possibility that a small proportion of the peptide that were studying had spontaneously dimerized in vitro after synthesis, and that some of the striking functional activity that we were measuring was due to steric hindrance of the dimerized peptide. Although dimerization would have little effect on our conjugates because the presence of a single cysteine could only form a single peptide-IgG link, this could be an important issue in designing more potent peptides or more efficacious peptide-IgG conjugates. Furthermore, we wished to state for the purposes of the article that we had conclusively identified the active LPS binding moiety of LBP.

To directly study this issue, we generated new sets of peptides spanning this region (LBP86-102, LBP86-106, LBP94-102, LBP94-106) with and without a terminal cysteine. We evaluated this new set of peptides in all of our binding and neutralization assays. It soon became apparent that there was something different with these peptides. They had only minimal and erratic activity and they were poorly soluble. We repeated the synthesis, and had similar results. A very frustrating six to eight weeks was spent working at all hours evaluating the peptides and seeking the problem. Finally, we discovered that the cleavage solution used to elute the peptides from the synthesis resin had been altered. We yet again resynthesized the peptides, this time using the old cleavage solution. We also synthesized the same peptides on a different peptide synthesizer, and submitted samples of both sets of peptides for mass spectrophotometer analysis for purity. This time we obtained decent binding and neutralization with both new sets of peptides. Our results suggested, but were not conclusive, that there may be some dimerization of the peptides with the terminal cysteine, that the LBP86-102 peptides with cysteine were somewhat more active in blocking LPS-CD14 activity than the same sequence without cysteine, and that the LBP86-106 sequences retained blocking activity in the absence of cysteine. These data suggested that the LBP86-102 sequence is needed for binding, but that additions on the carboxy tail might increase blocking activity, perhaps by adding steric hindrance.

To definitively test this hypothesis, we are constructing the following peptides:

LBP86-102C
LBPC102-86 (completely reversed sequence with cysteine)
LBP86-106C
LBP86-102-106-105-104-103C (LBP86-102 plus last four amino acids of 86-106 in reversed order, with C)

Each of the above peptides are being generated to be reduced, deliberately dimerized, and treated by a blocking reagent to prevent dimerization.

These experiments are in progress. We plan to assess these peptides in all of our assays. We hope that they will directly and definitively address the issue of dimerization, and whether the additional 102-106 tail is functioning not by increasing binding, but by blocking LBP-CD14 interactions. The reversed sequences will have the identical charge and hydrophobicity, but should have less activity because of the scrambled sequence.

In summary, it has been a frustrating time, in particular because the article on these sequences was completely written and was ready to submit in June. However, rather than get it wrong we held the article. We *think* that we have gained insight on the mechanism of the active LBP peptides. We hope we are not scooped on the work, and plan to submit the article the moment this last set of experiments is out.

B. Progress on Specific aim #2

As noted in the last report, we have not worked on identifying the LPS binding site of BPI. This work has been overshadowed by the other work in progress on LBP.

C. Progress on Specific aims #3 and #4

Two issues that will be important for the final peptide-IgG conjugate will be to optimize the number of peptides/IgG and the technical aspects of the peptide/IgG link.

1. Study of the number of peptides/IgG

We have developed both LBP and CAP18 conjugates with varying numbers of peptide/IgG by altering the molar concentration of the peptide during the coupling procedure. We next compared the functional ability of the different conjugates for the ability to bind and neutralize LPS and kill Gram-negative bacteria. These experiments indicate clearly that the activity of the conjugate to bind, neutralize, and kill Gram-negative organisms (in the case of CAP18-IgG conjugates) is directly proportional to the number of copies of peptide/IgG. Accordingly, conjugates with 4 or more peptides/IgG are the most active. Future in vivo experiments will need to assess if there is a downside to conjugates with many peptides/IgG with respect to clearance, stability, or toxicity. Cost may also be a factor. At this stage, conjugates with higher number of peptides are exponentially more expensive to produce because ever higher molar concentrations of peptide are needed at the time of coupling to gain relatively small increases in the number of peptides/IgG. If eventually a conjugate comes into clinical use, a molecular approach to production would likely be needed in which the additional cost of adding more peptides/IgG would be minimal.

2. Study of the optimal heterobifunctional linker

We have started to study the stability of the conjugates in buffer and blood. We began our work using conjugates made with the bifunctional linker, SPDP. At the time of the last report, we had started to study the use of a different, more stable heterobifunctional linker, SMPT. A goal for this trimester was study to the stability of conjugates made with these different linkers in whole blood. SMPT has the advantage that the disulfide bond has large molecular weight groups around the disufide bond between the peptide and IgG, thus diminishing reduction of the bond in vivo.

Over the last four months we have generated several batches of LBP(1-102-IgG and CAP18-IgG with both SPDP and SMPT and evaluated their binding affinity and stability in whole blood. The conjugates were preincubated in 20%

whole rabbit blood for varying times up to 24 hours, after which their ability to capture LPS was assessed by adding tritiated LPS followed 30 minutes later by magnetic beads coupled to rabbit IgG directed to human IgG. The beads were then magnetically separated and counted for captured radioactivity. A strength of this system is that it measures the ability of the conjugates to capture LPS in whole blood. Although because of the problems outlined above these experiments are still in progress for the LBP-IgG conjugates, it appears that for the rAPIB IgG conjugates there is an early loss of activity in blood over 15-30 minutes, and then a very slow decline over 24 hours. AMVI coupled conjugates appear in some experiments to be slightly more stable. One of these experiments is illustrated in Figure 3.

There appear to us three likely possibilities for the loss of activity over time. First, the peptide could be cleaved from the IgG. Second, the peptide could be inactivated by binding to antonic blood proteins, thus blocking its ability to subsequently bind to LPS. Third, the entire conjugate could be phagocytosed into monocytes and LPS via the Fc receptor, so that there is less peptide IgG conjugate available to bind LPS. We have started to design experiments to distinguish between these possibilities. One experiment is to prepare cultures from peptide and peptide IgG conjugates in whole blood and then add LPS and assess the TNF response of the blood. Since both uncoupled free rAPIB and LBP peptides inhibit the LPS-induced TNF response alone, a simple cleavage of the peptide from the IgG would not be expected to alter the TNF inhibition. Thus, if the incubation of the peptides alone and conjugates in whole blood resulted in a progressive loss of TNF inhibition, this might be evidence that the peptide itself is blocked by binding to antonic substances or enzymatically altered. In the one experiment that we have performed so far with the rAPIB IgG conjugates (in which the TNF response was only investigated), the AMVI conjugate retained activity longer than either the AMVI conjugate or equal molar quantities of peptide alone (Figure 4). If confirmed, this experiment suggests to us that the AMVI conjugate is preferable for inhibition of LPS induced TNF, and that conjugation may to some extent protect the peptide from degradation in blood.

We are designing experiments to study the possibility that the conjugates are taken into MNC. We believe that this issue is relatively important because conjugates that are bound to LPS might increase the phagocytosis of LPS via Fc receptors, thus increasing clearance.

Finally, we have recently become aware that there is yet another heterobifunctional linker in the same family, SMCC. This linker was designed to form an extremely stable bond with IgG in blood. Thus, we plan to compare conjugates formed with this linker with conjugates with AMVI and BMPS.

D. Progress on Specific Aim #3 :

As per our expected timetable, we have not yet started these specific aims.

II. New knowledge since the last award

1. LBP peptides with a cysteine on the carboxy terminal may be more active than similar peptides lacking a cysteine, and this may be due to dimerization of the peptide to create a double copy of the peptide. LBP peptides 86-109 may be

equally or more active than LHRH6-IgG. Experiments are in progress to pinpoint the optimal sequence for the conjugates.

2. Preliminary experiments suggest that SMPT linked peptide-IgG conjugates are equally active and slightly more stable in blood than MBBP-linked conjugates.

3. The activity of tAP1H-IgG and LHP-IgG with respect to the binding and neutralization of LHR is directly proportional to the number of peptides/IgG. On a molar basis, the present sequences of the tAP1H peptides alone and in the conjugated form are slightly more active than the present sequences of hLHP peptides alone and in the conjugated form.

4. Incubation of tAP1H IgG conjugates in 20% whole blood results in an rapid (1% 30 minute) loss of binding activity, followed by a slow decline in activity over 24 hours. The reasons for the early and later loss of binding activity is at the present unknown. SMPT conjugates are slightly more stable than MBBP conjugates. These experiments have preceded work with the LHP conjugates because we are awaiting a decision on the optimum sequence of LHP to work with definitively.

III. Technical problems

As outlined in A above, we had a major and very frustrating technical problem in the synthesis of two batches of synthetic peptides that resulted in insoluble peptides of poor quality. We believe that the problem related to a change in the elution procedure used to cleave the peptides from the resin upon which they were synthesized. The problem appears to be solved by returning to earlier method that had been utilized, and we believe that we are back on track. We have had no other technical problems.

IV. Future directions

1. A dominant issue that we believe is crucial to resolve is to identify the exact LHP sequence that will be optimal to conjugate to IgG. At present the options are hLHP1A IgG, hLHP2B IgG, and hLHP2A IgG. Our prime candidate was hLHP2A IgG. However, as noted above more recent experiments using peptides alone suggested that hLHP2A IgG may neutralize better. If any coupling on the carboxy terminal increases neutralization by providing steric hindrance, the additional 4 amino acids may not be much of an advantage for the development of conjugates. To resolve this issue, we have designed the peptides outlined in A above.

We would like to begin to focus on a single LHP sequence and conjugate in order to proceed with the development of a large lot that can be carefully evaluated in vivo.

2. A related issue will be to resolve as best as possible whether some of the activity of the previous sequences that we had made that had a terminal cysteine is due to dimerization. Since the LHP conjugates that we have made with hLHP1A IgG and hLHP2B IgG bind LHR, this issue is not directly relevant to the work with the conjugates. However, it will be important to solve this question rapidly in order to publish our data on the active binding site of LHP.

3. We hope to complete our studies of the stability of the conjugates in whole blood in the next 2 months. We plan to compare the different heterobifunc-

tional linkers mentioned above. The two assays that we will focus on regarding stability will be the ability to bind LPS over time and the ability to block LPS-induced TNF over time.

4. An issue related to #3 above that seems important to study is whether the conjugates are taken into WBC. It is possible that one reason that the activity of the conjugates slowly diminish over 24 hours in whole blood is that they are taken into cells. Issues that we will begin to study are:

a. are the conjugates taken into WBC in the bloodstream?
b. if so, is the uptake via Fc receptors?
c. is uptake increased if LPS is bound to the conjugate? Do the conjugates increase phagocytosis of LPS?

5. We plan to begin experiments studying the ability of the conjugates to bind and neutralize bacteria (as opposed to purified LPS).

6. A goal that we hope to get to in the next trimester is to generate peptides with ^{14}C to aid in the characterization of the conjugates and their stability and clearance in vivo.

V. Publications

The following articles are in press or submitted.

1. Kloczewiak M, Black KM, Loiselle P, Cavaillon JM, Wainwright N, Warren HS. Synthetic peptides that mimic the binding site of horseshoe crab anti-lipopolysaccharide factor. In press, Dec. 1994, J. Infectious Disease.

2. Warren HS, Black KM, Loiselle PL. Range and distribution of natural antibodies to the O-antigen of lipopolysaccharides in human plasma. Submitted. (This article was funded in part by the preceding Navy grant).

As noted above, we held submission of the following article about the binding site of LBP pending definitive conclusions regarding the issues relating to dimerization, length, and steric hindrance, and structure-function (binding-neutralization) relationships.

3. Warren HS, Cavaillon JM, Loiselle P, Ge Y, Black K, Zanzot E, Fitting C, Golenbock D, Vermeulen HW, Ezzell R, Kloczewiak M. Identification of a major LPS binding site of lipopolysaccharide binding protein.

We are in the process of preparing the first article on peptide-IgG conjugates. This article will describe the concept and our early conjugates using TAL7 peptides coupled to murine and human IgG. We plan to follow this article subsequent articles on CAP18-IgG conjugates and LBP-IgG conjugates (binding and neutralization data). The data and tables for each of these articles is complete and is awaiting drafting of the manuscripts.

VI. Legends to figures

Figure 1. Stability of CAP18-IgG conjugates in 20% whole blood over time. Ten ug/ml of CAP18-IgG conjugates made with SPDP or SMPT were preincubated in 20% whole rabbit blood for varying times. Two ug/ml tritiated E. coli 025 LPS were then added. Thirty minutes later, an excess of rabbit anti-human IgG coupled to magnetic beads was added. The beads with the captured conjugates with bound ^{3}H-LPS were then magnetically separated and the percent LPS captured assessed by liquid scintillation counting. Identical experiments with LBP-IgG conjugates are awaiting construction of conjugates with LBP with the finally selected LBP sequence.

Figure 2. Stability of CAP18 peptide and CAP18 conjugates made with SMPT or SPDP in 20% whole blood as assessed by inhibition of LPS-induced TNF production. Five ug/ml normal IgG or CAP18-IgG conjugates or equal molar quantities of CAP 18 peptide (0.27 ug/ml) were preincubated in 20% normal human blood for different times followed by the addition of 100 ng/ml LPS. Four hours later, the plasma was separated and assessed for TNF by bioassay. Longer incubation times are not possible because of the spontaneous induction of TNF. In this preliminary experiment the SMPT version of the CAP18-IgG conjugate inhibited after 90 minutes incubation in whole blood, whereas equimolar CAP18 peptide and the SPDP version of the conjugate were inactivated over time. Identical experiments with LBP-IgG conjugates are awaiting construction of conjugates with LBP with the finally selected LBP sequence.

Figure 1.

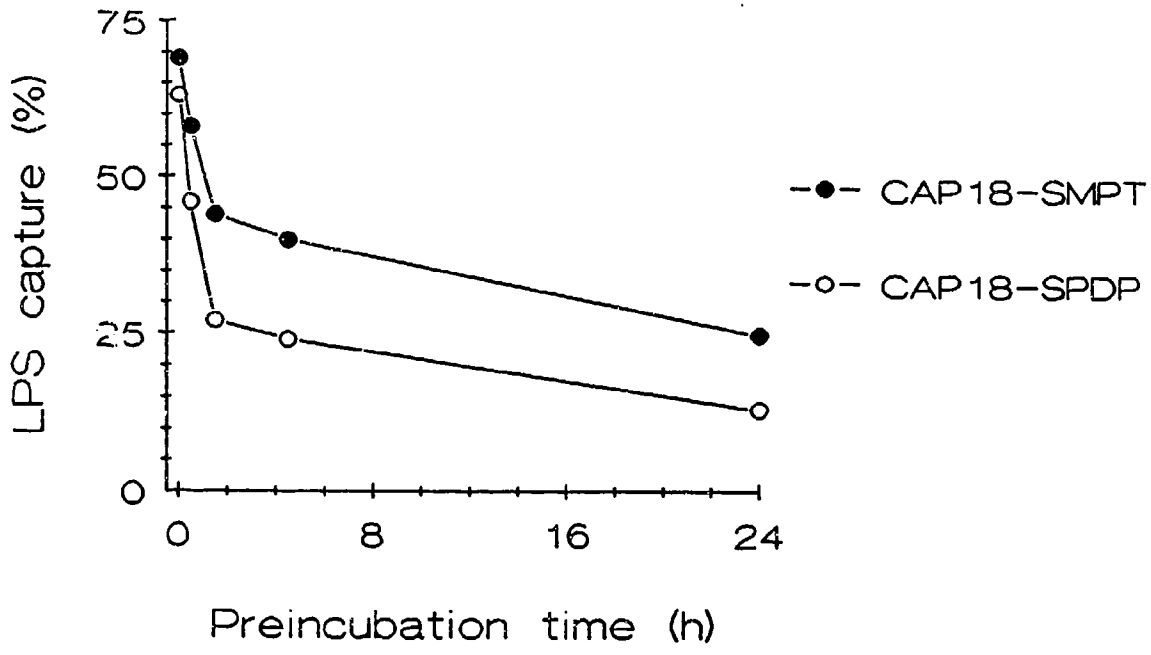

Figure 2.